natural product chemists for the isolation and purification of unstable bioactive materials from complex matrices. The technique deserves more attention from analytical chemists confronted with the separation of complex mixtures, synthetic chemists facing purification of non-crystalline products, diastereomers or optical resolution of racemates, and physical chemists seeking new approaches to the study of solvent interactions or the measurement of chemical properties such as partition coefficients or dissociation constants. Continuing study of new applications of CCC in several laboratories, as well as the commercial development of more reliable and user-friendly apparatus, promises to extend the use of CCC in the foreseeable future.

CCC Literature

References to CCC applications are found throughout the analytical and natural product literature and in the monographs cited below. A large number of references are found in *Journal of Chromatography* and in *Journal of Liquid Chromatography and Related Techniques*, many of which are collected in periodic special issues of these journals, the latest of which contains over 20 articles. A list of earlier special issues can be found in Conway (1995).

See also: **III/Alkaloids:** High-speed Countercurrent Chromatography. **Antibiotics:** High-speed Countercurrent Chromatography. **Chiral Separations:** Countercurrent Chromatography. **Dyes:** High-speed Countercurrent Chromatography. **Ion Analysis:** High-speed Countercurrent Chromatography. **Natural Products:** High-speed Countercurrent Chromatography. **Proteins:** High-speed Countercurrent Chromatography.

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Detectors: Laser Light Scattering

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Introduction

There are two types of light scattering detectors used in liquid chromatography, the evaporative light scattering detector and the liquid light scattering detector. The former evaporates the solvent from the column eluent in a gas stream and measures the light scattered by the residual solute particles; this type of detector is described elsewhere in this encyclopedia. The second, the liquid light scattering detector, senses the actual solute molecules themselves by light scattering measurements. Liquids containing large molecules such as synthetic polymers, biopolymers, e.g. polypeptides, proteins and polysaccharides, scatter light and, providing the incident light is strong enough, the scattered light can be sensed and used to detect the presence of the solute. In practice the column eluent is allowed to flow though a cell through which passes a high intensity beam of light. The light source is usually a parallel beam laser (light amplification by

the stimulated emission of radiation) that generates light at an appropriate wavelength for measurement. The scattered light is viewed at a specific angle by a photosensor, the output of which is electronically modified and passed to a potentiometric recorder or, more probably, to a computer data acquisition system.

Alternative Light Scattering Detectors

Two basic types of liquid light scattering detectors have been developed and made commercially available, each having its unique advantages and disadvantages. The two forms of the detector: the low angle laser light scattering (LALLS) detector and the multiple angle laser light scattering (MALLS) detector. Both devices are used extensively in polymer analysis. The multiple angle laser light scattering detector is somewhat more versatile and, under the right conditions, can also provide molecular dimensions as well as molecular masses. As would be expected from its name, in the low angle laser light scattering detector, the intensity of the scattered light is measured at a very small angle to the path of the incident light (virtually 0°). Under these conditions, the signal can also receive light that has been scattered by contaminating particulate matter that is always present in the eluent. This extra light source can contribute considerable noise to the signal, which, in turn, will reduce the detector sensitivity. Discussions on these aspects of the different detectors have been given by, P.J. Wyatt and some early experimental results reported by D.T. Phillips.

From the work of Rayleigh, the ratio of the intensity of the light scattered at an angle (ϕ), (I_ϕ) to the intensity of the incident light (I_0), is given by the following equation:

$$\frac{I_\phi}{I_0} = \alpha\omega R_\phi \qquad [1]$$

where α is the attenuation constant, ω is a function of the refractive index, and R_ϕ is Rayleigh's constant.

Thus, the Rayleigh constant can be extracted from the above equation giving:

$$R_\phi = \frac{I_\phi}{\alpha\omega I_0} \qquad [2]$$

Now, the relative molecular mass (M_r) of the solute that is scattering the light is, in turn, related to the Rayleigh factor by the following expression:

$$M_r = \frac{R_\phi}{c(K - 2A_2R_\phi)} \qquad [3]$$

where c is the concentration of the solute, A_2 is a function of polymer–polymer interactions, and K is the polymer optical constant.

Substituting for R_ϕ in eqns [3] from [2]:

$$M_r = \frac{I_\phi/\alpha\omega I_0}{c(K - 2B_2(I_\phi/\alpha\omega I_0))} = \frac{I_0}{c(\alpha\omega I_0 K - 2B_2 I\phi)} \qquad [4]$$

where:

$$K = \frac{2\pi^2\eta^2}{\lambda^4 N(\mathrm{d}\eta/\mathrm{d}c)^2} \qquad [5]$$

and η is the solvent refractive index, λ is the wavelength of the light in vacuum, and N is the Avogadro number.

Eqn [4] gives the basic relationship between the relative molecular mass of the scattering material, the intensity of the scattered light and the physical properties of the materials and equipment that are being employed. Unfortunately, eqn [4], includes a number of constants, the magnitude of which can be extremely difficult, if not impossible, to determine accurately. Consequently, an alternative procedure must be adopted to handle the data provided by the intensity of the scattered light and the angle at which it is measured. In practice, a simple graphical procedure is adopted to determine the relative molecular mass of the solute that avoids the need to determine all the pertinent constants.

Rearranging eqn [3]:

$$\frac{1}{M_r} = \frac{c(K - 2A_2R_\phi)}{R_\phi} = \frac{cK}{R_\phi} - 2cA_2$$

or:

$$\frac{cK}{R_\phi} = 2cA_2 + \frac{1}{M_r} \qquad [6]$$

This arrangement provides a convenient linear relationship between the important variables that can be measured. Thus c, K and R_ϕ are either known or can all be calculated from known data and calibration light scattering measurements; consequently, by plotting (cK/R_ϕ) against c a straight line will be produced with the intercept being ($1/M_r$).

It follows that a value for the relative molecular mass of the eluted polymer can also be estimated.

Low Angle Laser Light Scattering Detector

The optical system of a commercial low angle laser light scattering detector is shown diagramatically in

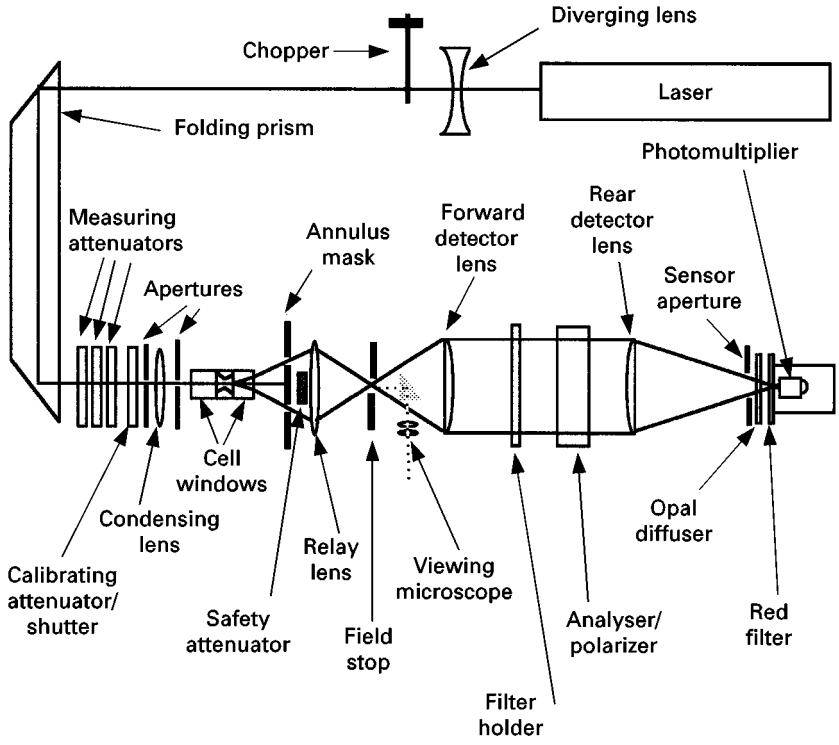

Figure 1 Optical diagram of a low angle laser light scattering detector (LALLS). (Reproduced by permission of LDC Analytical, Thermo Instruments Corporation.)

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Figure 1. Owing to the length of the laser light generator, the instrument can become extremely bulky so certain optical space-saving arrangements need to be adopted. To conserve space and make the optical system compact, a folding prism system is used that allows the optical system to be contained to a reasonable size and still accommodate the length of the laser generator.

Light from the laser source passes through diverging lens, then through a chopper, and finally through the folding prism. The laser beam then passes out of the folding prism, through some measuring attenuators, then through a calibrating attenuator shutter and finally through the cell. Between the cell and the relay lens is placed an annular mask that only allows light scattered in the cell at a low angle to pass to the relay lens. Between the annular shutter and the relay lens is placed a safety attenuator that ensures that none of the laser light can reach the photomultiplier, which would cause severe damage. The scattered light is focused through a field stop onto the forward detector lens. For convenience. a prism is placed between the field stop and the forward detector lens, allowing the scattered light to be viewed through a microscope. A filter holder and an analyser/polarizer is placed between the forward detector lens and the rear detector lens. Finally, the scattered light is focused through the sensor aperture on to an opal diffuser that spreads the scattered light through a red filter and onto the sensor plate of a photomultiplier. The device is conveniently operated in series with a refractive index detector in order to coincidentally measure the refractive index of the eluent.

It is clear from eqn [5] that in order to calculate K the refractive index of the solute must also be known. It is also seen from eqn [4] that an estimate of the relative molecular mass of a solute can be obtained from the intercept $(1/M_r)$ of the graph relating (cK/R_ϕ) to the solute concentration c, as shown in **Figure 2**. The concentration is usually determined from the output of the refractive index detector from prior calibration.

The overall sensitivity (minimum detectable concentration) of the detector appears to be very similar to that of the refractive index detector (i.e. about 1×10^{-6} g mL^{-1} at a signal-to-noise ratio of 2) and would seem to have about the same linearity and linearity range (i.e. $0.97 > r > 1.03$ over a concentration range of 2–3 orders of magnitude). However, the most important characteristic of this detector is not its propensity for accurate quantitative analysis but its proficiency in providing relative molecular mass for extremely large molecules.

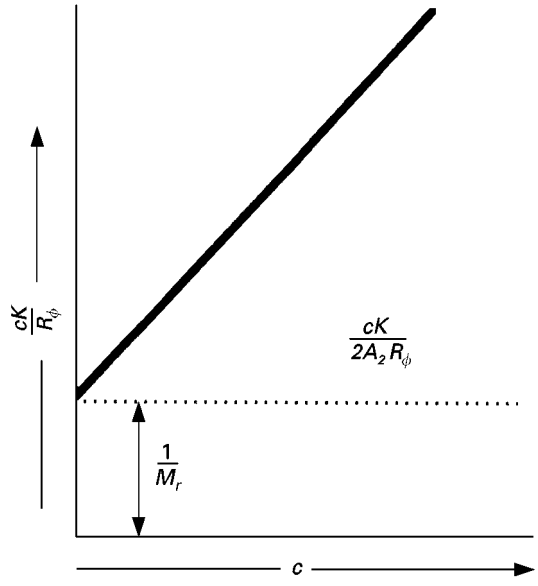

Figure 2 Determination of relative molecular mass from low angle light scattering measurements.

An example of the use of the low angle laser light scattering detector to monitor the separation of bovine serum albumin monomer, antibovine serum albumin and bovine serum albumin complex is shown in **Figure 3**. The relative molecular masses of the three components as measured by the LALLS detector were, bovine serum albumin monomer, 66 700 (literature value 66 000), anti-bovine serum albumin, 150 800 (literature value 150 000) and the bovine serum albumin complex 297 300. It is seen that fairly accurate estimates of relative molecular mass can be achieved by this type of detector. The column used was the G 3000 SWXL and the mobile phase a phosphate saline solution buffered at pH 7.1–7.2. The flow rate was 0.4 mL min^{-1} and the sample volume was 100 μL. The UV detector was operated at 280 nm.

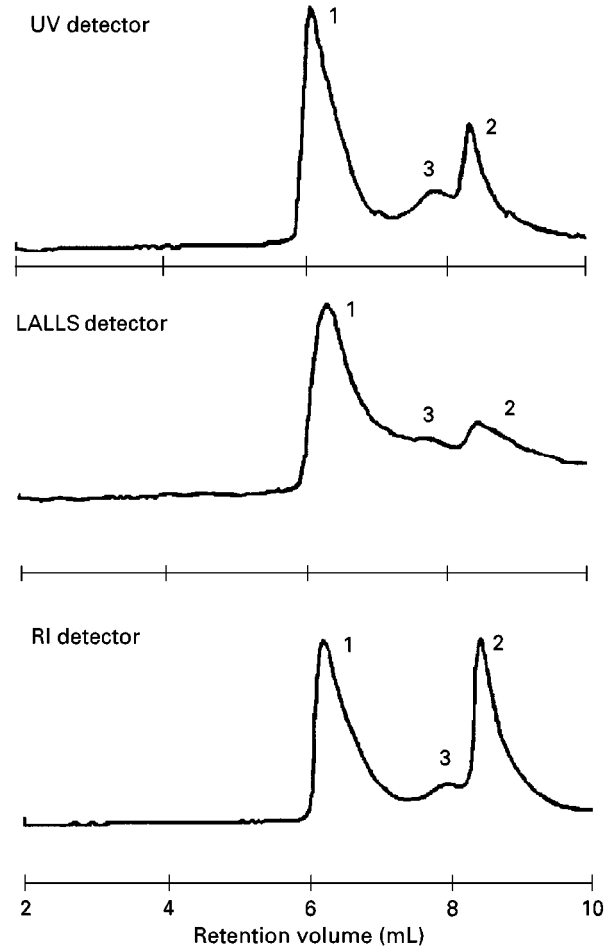

Figure 3 Separation of antibovine serum albumin and some bovine serum albumin complexes. Peaks: 1, antibovine serum albumin; 2, bovine serum albumin monomer; 3, bovine serum albumin complex. (Reproduced by permission of Qian RL, Mhatre R and Krull IS, 1997.)

Multiple Angle Laser Light Scattering (MALLS) Detector

The multiple angle laser light scattering detector differs significantly from the low angle laser light scattering detector in that scattering measurements with this device are made at a number of different angles, none of which are close to the incident light. This reduces, in fact almost eliminates, the problem associated with scattering from particulate contaminants in the sample. In addition, measuring the scattered light simultaneously at a number of different angles allows the root-mean-square (rms) of the molecular radius $\langle r^2 \rangle^{1/2}$ of the polymer to be calculated in addition to its relative molecular mass.

The relationship that is used to process the data from this detector is as follows:

$$\frac{cK}{R_\phi} = a\langle r^2 \rangle^{1/2} \sin(\phi)^2 + bM_r \qquad [7]$$

where the symbols have the meanings previously defined.

In fact, detailed examination of the theory of light scattering at larger angles can provide explicit functions for the constants a and b. However, in practice, values for these constants are usually obtained from measurements made on calibrating substances of known relative molecular mass and molecular radii. Furthermore, in any practical device each photocell (used to sense the light at the different scattered angles) will not have precisely the same response to light of low level intensity. Consequently, calibration

procedures are also necessary to take the different response of the individual sensors into account to provide appropriate correction factors.

The number of different angles at which the scattered light is measured differs widely with different instruments, and commercial equipment that measure the intensity of the scattered light at as many as 16 different angles are available. It is clear that the greater the number of data points taken at different angles, the more precise and accurate the results will be. A diagram of a relatively simple commercial (MALLS) detection system which measures the light scattered at only three different angles is shown in **Figure 4**. The device is very simple – it contains no mirrors, prisms or moving parts and is designed such that the light paths are direct and there is no need to use an optically 'folded' light system. It is seen Figure 4 that light passes from the laser (wavelength 690 nm) directly through a sensor cell. The scattered light passes from the centre of the cell through three narrow channels to three different photocells, set at 45° and 90° and 135° to the incident light. Thus, scattered light is continuously sampled at three different angles during the passage of the solute through the cell.

A continuous analogue output is provided from the 90° sensor for monitoring purposes and, in the particular system described, all the sensors are sampled every 2 s. The relative molecular mass range is claimed to extend from 10^3 to 10^6 and the rms radii from 10 to 50 nm. The total cell volume appears to be about 3 μL and the scattering volume is as little as 0.02 μL. The detector has a sensitivity, which is defined by the manufacturers, in terms of the minimum detectable excess Rayleigh ratio of $5 \times 10^{-8}\,\mathrm{cm}^{-1}$. This sensitivity is difficult to translate into normal concentration units but appears to be very similar to that of the refractive index detector, which is equivalent to a minimum detectable concentration of about $10^{-6}\,\mathrm{g\,mL}^{-1}$.

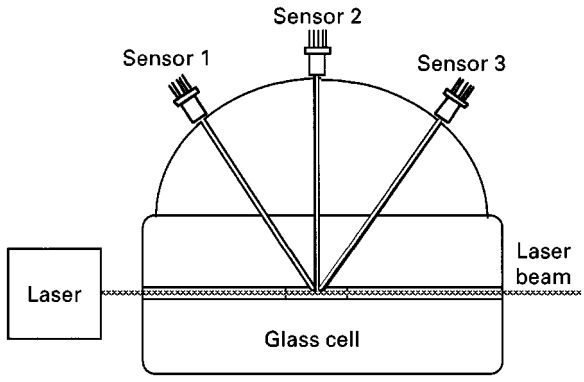

Figure 4 The multiple angle laser light scattering detector (miniDawn®). (Reproduced by permission of Wyatt Technology Corporation.)

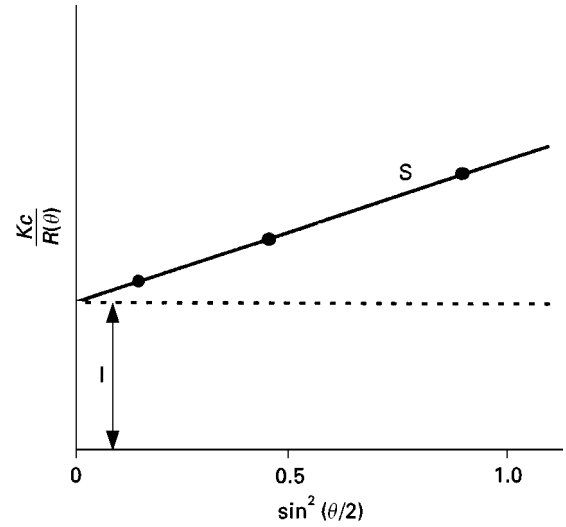

Figure 5 Calibration curves for the multi angle light scattering detector. Intercept (I) gives a value for the relative molecular mass; slope (S) give a value for the rms molecular radius.

The relationship between the intensity of the scattered light, the scattering angle and the molecular properties, are given by the following equation:

$$\frac{cK}{R_\phi} = 2cA_2 + \frac{1}{M_r P(\phi)}$$

where $P(\phi)$ describes the dependence of the scattered light on the angle of scatter and the other symbols have the meanings previously attributed to them.

In fact, the relationship between the angle of scattering, θ, the relative molecular mass and the rms molecular radius of the solute is obtained using eqn [7]. Employing appropriate reference materials, graphs of the form shown in **Figure 5** can be constructed to evaluate constants a and b and thus permit the measurement of the relative molecular mass and molecular radius of unknown substances. This detecting system can be extremely valuable when dealing with unknown biopolymers where little or no evidence is available as to their mass or size and the use of the mass spectrometer is prohibited by either their mass or thermal instability.

The separation of bovine serum albumin (BSA), lysozyme, bradykinin and leucine enkaphalin monitored by the multiangle light scattering detector and the refractive index detector in **Figure 6**. The high response of the multiangle light scattering detector to the high relative molecular mass BSA is clearly demonstrated. The relative molecular mass measurements made on the solutes are shown in

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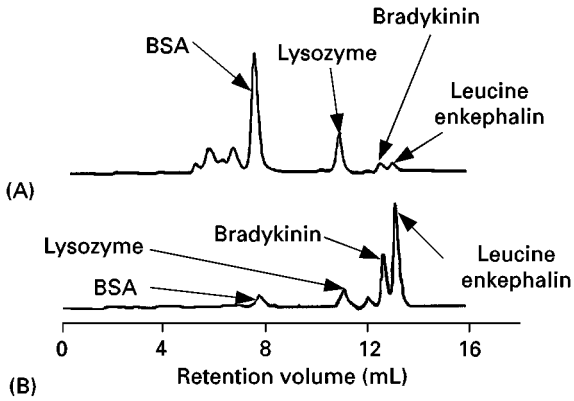

Figure 6 Chromatograms obtained simultaneously from the multiangle light scattering detector (A) and the refractive index detector (B).

Table 1 Peptide and protein mass values from the multiangle light scattering detector

Peak	*Solute*	M_r (*sequencing*)	M_r (*measured*)
1	BSA	67 000	64 300 ± 700
2	Lysozyme	14 300	14 600 ± 300
3	Bradykinin	1 060	1 090 ± 10
4	Leucine-enkephalin	556	592 ± 6

Table 1, which also includes relative molecular mass data obtained from sequencing the solutes.

It is seen that fairly accurate values for relative molecular mass can be obtained for the larger molecules, which can be extremely useful when dealing with completely unknown biopolymers. The errors involved are significantly greater for the materials of smaller relative molecular mass because the response (the amount of light scattered) is much less, and the output signal is much closer to the noise level of the sensing system. Further discussion of these types of detector are furnished in references provided in the Further Reading section.

See also: **II/Chromatography: Liquid:**Detectors: Evaporative Light Scattering. **Chromatography:** Protein Separation. **Appendix 1/Essential Guides for Isolation/Purification of Drug Metabolites. Essential Guides for Isolation/Purification of Enzymes and Proteins.**

Further Reading

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Hydrodynamic Chromatography

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Summary

Hydrodynamic chromatography (HDC) is one of the many techniques for particle size determination in the micron range. It has some similarities with size exclusion chromatography and field flow fractionation, but needs only one phase and one field. The main advantages are the separation of species according to size only, rapidity of measurement in the untreated medium and ease of operation of equipment. Variation in operating parameters allows a considerable range of possible applications. Disadvantages are low resolution, necessity for peak dispersion correction and calibration for signal intensity according to size. It has many applications in latex production and quality control.

Definition and General Features

Definitions

Particles may be separated according to their size by several techniques: HDC is one of them. This interesting rapid method (about 10 min) separates and sizes solutes or particulates in the micron range (0.030–60 μm) at a high dilution, without being